

**Title:**

Potential of adenovirus-mediated REIC/Dkk-3 gene therapy for use in the treatment of pancreatic cancer

**Short title: REIC gene therapy for pancreatic cancer****The names of the authors and their affiliations, addresses:**

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## Abstract

**Background and Aim:** The reduced expression in immortalized cells REIC/Dkk-3 gene, tumor suppressor gene, is downregulated in various malignant tumors. In a prostate cancer study, an adenovirus vector carrying the REIC/Dkk-3 gene (Ad-REIC) induces apoptosis. In the current study, we examined the effects of REIC/Dkk-3 gene therapy in pancreatic cancer.

**Methods:** REIC/Dkk-3 expression was assessed by immunoblotting and immunohistochemistry in the pancreatic cancer cell lines (ASPC1, MIAPaCa2, Panc1, BxPC3, SUIT-2, KLM1 and T3M4) and pancreatic cancer tissues. The Ad-REIC agent was used to investigate the apoptotic effect *in vitro* and anti-tumor effects *in vivo*. We also assessed the therapeutic effects of Ad-REIC therapy with gemcitabine.

**Results:** The REIC/Dkk-3 expression was lost in the pancreatic cancer cell lines, and decreased in pancreatic cancer tissues. Ad-REIC induced apoptosis and inhibited cell growth in the ASPC1 and MIAPaCa2 lines *in vitro*, and Ad-REIC inhibited tumor growth in the mouse xenograft model using ASPC1 cells. The anti-tumor effect was further enhanced in combination with gemcitabine. This synergistic effect may be caused by the suppression of autophagy via the enhancement of mTOR signaling.

**Conclusions:** Ad-REIC induces apoptosis and inhibits tumor growth in pancreatic

cancer cell lines. REIC/Dkk-3 gene therapy is an attractive therapeutic tool for pancreatic cancer.

**Key words:** gene therapy, Dickkopf-related protein, apoptosis, mTOR pathway, autophagy

**Abbreviations:** GEM, gemcitabine; ER, endoplasmic reticulum

## Introduction

Pancreatic cancer is a cancer with a poor prognosis and is the fourth largest cause of cancer death worldwide [1-3]. Currently, treatment with gemcitabine (GEM) is the standard therapy for unresectable pancreatic cancer. However, the overall 5-year survival rate of pancreatic cancer is less than 5% [4]. In order to achieve a breakthrough, various gene therapies for pancreatic cancer have been studied and applied in clinical applications [5-9].

Reduced expression in immortalized cells (REIC), also known as the dickkopf 3 (Dkk-3) gene, was originally identified to be a tumor suppressor gene in association with the immortalization of normal human fibroblasts and is downregulated in various cancers [10]. The REIC/Dkk-3 expression is lost or decreased in various cancers, while the overexpression of REIC/Dkk-3 caused by adenovirus vectors induces cancer cell death [11-16]. The overexpression of REIC/Dkk-3 induces endoplasmic reticulum (ER) stress evoked by unfolded proteins [13] that activate c-Jun N-terminal kinase (JNK) and stimulate apoptosis. Interestingly, apoptosis occurs in only cancer cells. Normal cells evade apoptosis and induce the immune response due to the induction of interleukin-7 [17].

In the current study, we evaluated the therapeutic potential of REIC/Dkk-3 gene

therapy to overcome the inferior prognosis observed in patients with pancreatic cancer.

## **Materials and Methods**

### ***Cell lines and cell culture:***

The pancreatic cancer cell lines ASPC1, MIAPaCa2, Panc1 and BxPC3 were obtained from DS Pharma Biochemical Co., Ltd. (Osaka, Japan), SUIT-2 was obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan) and KLM1 and T3M4 were obtained from the Riken cell bank (Ibaraki, Japan). ASPC1, MIAPaCa2 and Panc1 were maintained in Dulbecco's modified Eagle's medium DMEM (Invitrogen, Carlsbad, CA), BxPC3, SUIT2 and KLM1 were maintained in RPMI- 1640 (Sigma, St. Louis, MO) and T3M4 was maintained in F10-HAM (Sigma). The media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma), 1% non-essential amino acid (Sigma), 1% penicillin/streptomycin solution (Sigma) and amphotericin B (0.5 µg/ml). The cells were cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air and quiesced at subconfluence under restricted serum conditions with 0.1% dialyzed FBS for 24 hours before the experiment.

### ***Immunoblot analysis***

Cells were plated into six-well tissue culture plastic dishes and grown to confluence. The cells were washed twice with cold phosphate-buffered saline (PBS) and lysed with

PRO-PREP protein extraction solution (iNtRON Biotechnology, Gyeonggi, Korea). The samples were resolved via SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Immobilon-P™ membranes (Millipore Corporation, Bedford, MA). The membranes were blocked with Tris buffered saline with Tween 20 (Sigma) (TBS-T) buffer containing 1% bovine serum albumin for one hour and then incubated with antibodies against human phospho-c-Jun (Ser73), human phospho-stress-activated protein kinase/Jun amino-terminal kinase (SAPK/JNK) (Thr183/Tyr185), phospho-mammalian target of rapamycin (p-mTOR) (Ser2448) (Ser2481), phospho-p70S6 kinase (p-p70S6K) (Thr389), human  $\beta$ -gal(LacZ), human  $\beta$ -actin (Cell Signaling Technology, Danvers, MA) and human phospho-inositol-requiring enzyme 1 $\alpha$  (IRE1 $\alpha$ ) (S724) (Abcam, Cambridge, MA).

### ***Pancreatic cancer tissues and immunohistochemistry***

Pancreatic cancer and adjacent tissues were obtained from 29 patients who underwent pancreaticoduodenectomy or distal pancreatectomy. None of the patients received chemotherapy before surgery.

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded sections. The sections were dewaxed and dehydrated. Following rehydration, the endogenous



peroxidase activity was blocked for 30 minutes in a methanol solution containing 0.3% hydrogen peroxide. Following antigen retrieval in citrate buffer, the sections were blocked overnight at 4°C and then probed with anti-human REIC/Dkk-3 antibodies [14]. The primary antibody was detected with a biotinylated anti-mouse antibody (Dako Japan, Tokyo, Japan). The signal was amplified via avidin-biotin complex formation and developed with diaminobenzidine followed by counterstaining with hematoxylin, dehydration in alcohol and xylene and mounting. The percentage of positively stained tumor cells per field was calculated and categorized into three groups: (-), 0-10% positive; (+), 10-30%; (++) , 30-70%; and (+++) , >70% in reference to a previous report [14]. All sections were examined and scored independently by two observers without any prior knowledge regarding clinical background. All discrepancies in the evaluation of the REIC/Dkk-3 expression were reviewed, and a consensus was reached.

### ***Adenovirus vectors***

REIC/Dkk-3 was overexpressed using an adenovirus [13]. A full-length cDNA was integrated into a cosmid vector pAxCawt and transferred into an adenovirus vector according to the COS-TPC method (Takara Bio, Shiga, Japan). An adenovirus vector carrying the REIC/Dkk-3 gene (Ad-REIC) was created, and an adenovirus vector

carrying the LacZ gene (Ad-LacZ) was used as a control. A total of  $2 \times 10^5$  cells/well were seeded in flat-bottomed six-well plates, incubated for 24 hours and treated with Ad-LacZ or Ad-REIC at the indicated multiplicities of infection (MOI) in serum-free medium for one hour. The medium was then changed to flesh complete medium.

#### ***Assessment of cell proliferation and death***

A total of  $2 \times 10^5$  cells/well were seeded in flat-bottomed six-well plates, incubated for 24 hours and treated with Ad-LacZ or Ad-REIC at the indicated multiplicities of infection (MOI) in serum-free medium for one hour. The medium was then changed to flesh complete medium. After 24 hours of incubation, the cells were cultured for 72 hours with or without  $1 \mu\text{M}$  of gemcitabine (GEM) (Eli Lilly Co., Indianapolis, IN). Following the completion of the incubation period, Hoechst 33342 (Wako, Tokyo, Japan) was added to the medium at a concentration of  $2 \mu\text{g/ml}$ , and the cells were incubated in the dark for 10 minutes [16]. The number of apoptotic cells was counted under a microscope, and the percentage for each sample was determined (100 cells were judged per field, and the average percentage of apoptotic cells in five different fields was calculated). Cell viability was measured by counting the number of viable cells. The cells were detached with trypsin, stained with 0.4% trypan blue and counted using

an automated cell counter (Life technologies Japan, Tokyo, Japan).

### ***Autophagy assay***

We used the Cyto-ID Autophagy Detection kit (Enzo Life Science, Inc., NY, USA) to evaluate the autophagy of pancreatic cancer cells infected with Ad-REIC. After 48 hours of incubation, autophagy detection reagent and Hoechst33342 nuclear stain were added, and the results were evaluated using fluorescence microscopy. Rapamycin was used as an autophagy inducer. The cells were pretreated with 500  $\mu$ M of rapamycin for 12 hours. The number of cells exhibiting autophagy was counted under a fluorescence microscope, and the percentage for each sample was calculated (100 cells were judged per field, and the average percentage of cells exhibiting autophagy in five different fields was calculated).

### ***Pancreatic cancer xenografts in nude mice***

ASPC1 cells ( $2.0 \times 10^6$  in 100  $\mu$ l of PBS) were injected subcutaneously into the left flanks of 8-week-old BALB/c nude mice (SLC, Hamamatsu, Japan). One week after injection,  $1.0 \times 10^9$  plaque forming units of Ad-REIC or Ad-LacZ in 100  $\mu$ l of PBS was injected intra and peritumorally. The same volume of PBS was injected as a negative

control. Starting two days after the injection of Ad-LacZ or Ad-REIC, the mice were treated weekly with the i.p. administration of GEM or PBS for four weeks. The dose of gemcitabine was 80 mg/kg administered weekly. The tumor size was measured every week for four weeks after injection. The tumor volume was calculated using the following empirical formula:  $V = 1/2 \times [(\text{the shortest diameter})^2 \times (\text{the longest diameter})]$ . We assigned ten mice to each group. REIC/Dkk-3 protein expression was confirmed by the immunoblot analysis of the tumor tissues. The mice were sacrificed after 72 h from the treatment with each adenovirus vector (Ad-LacZ, Ad-REIC). The proteins were extracted from the tumor tissues with snap-frozen in liquid nitrogen with PRO-PREP protein extraction solution. The care and treatment of the mice were in accordance with the guidelines of the IACUC at the Okayama University Faculty of Medicine.

### ***Statistical analysis***

Correlations between the REIC expression and clinicopathological variables were analyzed using the  $\chi^2$  test. For the *in vitro* studies, each experiment was performed independently at least three times. Statistically significant differences were determined according to Student's *t*-test using the JMP10 software program (SAS Institute, Japan).



## Results

### *REIC/Dkk-3 expression in pancreatic cancer*

In the ASPC1 cell line treated with Ad-REIC, a positive control, REIC/Dkk-3 proteins were detected as major bands of 60 kDa in size. The REIC/Dkk-3 protein expression was assessed in several human pancreatic cancer cell lines (Fig. 1A). The REIC/Dkk-3 expression was absent in all pancreatic cancer cell lines (ASPC1, MIAPaCa2, Panc1, BxPC3, SUIT2, KLM1 and T3M4). REIC/Dkk-3 was detected in the normal pancreatic acinus, duct epithelial cells and cells in the islets of Langerhans; however, the expression of REIC/Dkk-3 was decreased in the cancer cells of the human pancreatic cancer tissues (Fig. 1B). Twenty-three cases (79.3%) exhibited a negative (-) signal for the REIC/Dkk-3 expression in the pancreatic cancer tissue. Four cases demonstrated a weak signal (+) for the REIC/Dkk-3 expression, while two cases displayed an intermediate signal (++). No cases showed a strong (+++) signal (Table 1). The REIC/Dkk-3 expression in the human pancreatic cancer cells tissues compared to that observed in the corresponding tumor-free sections. The islets of Langerhans, which are known to express REIC/Dkk-3 proteins [18], were used as a positive control. Seventeen cases (82.4%) showed a positive signal for the REIC/Dkk-3 expression in the tumor-free sections of the pancreatic tissue, while only three cases (17.6%) exhibited a

negative signal (Table 1).

### ***Cell proliferation and apoptosis***

As the REIC/Dkk-3 expression has been reported to induce apoptosis in prostate cancer cells [13], an MTT assay was performed to determine whether the REIC/Dkk-3 expression influences cell growth in the pancreatic cancer cell lines ASPC1 and MIAPaCa2. Gemcitabine was also used to determine whether REIC gene therapy increases the effects of standard systemic chemotherapy against pancreatic cancer. The degree of cell proliferation was decreased in the Ad-REIC (300 MOI)-treated cells (ASPC1:  $31.9 \pm 8.8\%$ , MIAPaCa2:  $39.7 \pm 11.6\%$ , ratio to the Ad-LacZ-treated cells,  $p < 0.05$ ) and Ad-REIC (300 MOI) with GEM-treated cells (ASPC1:  $29.1 \pm 6.8\%$ , MIAPaCa2:  $19.7 \pm 3.1\%$ , ratio to the Ad-LacZ-treated cells,  $p < 0.05$ ) (Fig. 2A). The effects of REIC/Dkk-3 were assessed in order to investigate whether the elicited growth suppression was due to an increase in cell death. Hoechst 33342 staining demonstrated that the rate of apoptosis in the cells treated with Ad-REIC (ASPC1:  $39.7 \pm 7.0\%$ , MIAPaCa2:  $36.1 \pm 5.7\%$ ) and Ad-REIC with GEM (ASPC1:  $57.3 \pm 8.5\%$ , MIAPaCa2:  $56.1 \pm 8.8\%$ ) was significantly higher than that of the cells treated with Ad-LacZ (ASPC1:  $9.3 \pm 0.7\%$ , MIAPaCa2:  $13.0 \pm 1.4\%$ ) and Ad-LacZ with GEM (ASPC1:  $23.0$

$\pm 3.5\%$ , MIAPaCa2:  $29.0 \pm 5.3\%$ ) (Fig. 2B).

***Ad-REIC induced apoptosis via the c-Jun signaling pathway and suppressed autophagy***

We confirmed that the level of apoptosis achieved through the c-Jun N-terminal kinase (JNK) pathway was enhanced by Ad-REIC using an immunoblot analysis (Fig. 3). This finding proves that REIC/Dkk-3 overexpression induces apoptosis by increasing ER stress in pancreatic cancer and other cancer cells [13-16]. In addition, the ER stress induced by Ad-REIC did not stimulate autophagy, but rather tended to suppress autophagy, although the difference was not statistically significant (Fig. 4). mTOR signaling, which is known to be a regulator of autophagy, was enhanced by Ad-REIC (Fig. 5).

***Mice xenograft models***

To determine whether Ad-REIC inhibits tumor growth *in vivo*, we constructed a mouse xenograft model using ASPC1 cells (Fig. 6A). Ad-REIC inhibited tumor growth by  $76.4 \pm 11.8\%$ , while control Ad-LacZ did not exert any significant effects on tumor growth ( $108.9 \pm 13.6\%$ ). Ad-REIC also inhibited tumor growth by  $70.9 \pm 14.8\%$



( $p < 0.05$ ) under the presence of GEM. The tumor growth in the Ad-REIC group was significantly suppressed compared to that observed in the Ad-LacZ group ( $p < 0.05$ ).

The efficiency of REIC/Dkk-3 gene transduction was shown with immunoblot analysis (Fig. 6B). The REIC/Dkk-3 protein was successfully expressed in the tumor tissues in Ad-REIC group and Ad-REIC+GEM group.

## Discussion

As the prognosis of patients with pancreatic cancer is poor [1-3], new therapies are needed to treat pancreatic cancer patients. Adenovirus-mediated gene therapy is a potential new method, and trials of this technique have been reported [19, 20]. In the current study, we assessed the possibility of Ad-REIC gene therapy for use in the treatment of pancreatic cancer.

REIC/Dkk-3 has been reported to be an inhibitor of tumor growth in various cancers [21] [13-16] due to its actions in interfering with Wnt signaling [22]. Fong, D. et al. reported that the Dkk-3 expression is correlated with the prognosis of patients with pancreatic cancer and that Dkk-3 overexpression inhibits tumor growth in pancreatic cancer cell lines [23]. As these results suggested that Ad-REIC is a promising gene for use in therapy for pancreatic cancer, we assessed the expression of REIC/Dkk-3 in pancreatic cancer tissues and evaluated the therapeutic *in vitro* and *in vivo* effects using adenovirus vectors. The REIC/Dkk-3 expression was decreased or lost in the pancreatic cancer cell lines (Fig. 1A) and pancreatic cancer tissues (Fig. 1B). As expected, REIC/Dkk-3 overexpression induced apoptosis and inhibited cell proliferation (Fig 2).

REIC/Dkk-3 overexpression induces apoptosis by increasing ER stress via the overproduction of REIC/Dkk-3 proteins in prostate cancer cells [13, 24]. In accordance

with the results observed for prostate cancer, in this study, the c-Jun N-terminal kinase pathway was activated by Ad-REIC in the pancreatic cancer cells (Fig. 3). These results suggest that Ad-REIC induces apoptosis by increasing ER stress in pancreatic cancer cells.

We also examined the effects of Ad-REIC in combination with chemotherapy, as GEM is commonly used for standard chemotherapy in patients with pancreatic cancer [25]. Onimaru M. et al. reported that GEM synergistically enhances the effects of adenovirus gene therapy [26]. In the present study, GEM enhanced the anti-tumor effects of REIC/Dkk-3 in the pancreatic cancer tissues (Figs. 2 and 6).

mTOR is known to be a strong suppressor of autophagy [27-29]. Autophagy is a catabolic membrane-trafficking process that is activated by various cellular stressors [30]. Autophagy has been reported to promote the survival of cancer cells and contribute to the development of tolerance to chemotherapy [30-32]. On the other hand, autophagy has been reported to suppress tumor growth [33, 34]. Although the effects of autophagy remain controversial, the inhibition of autophagy is believed to be beneficial for cancer therapy [35, 36]. ER stress has been reported to induce autophagy [37]; however, our results demonstrated that autophagy is not enhanced by Ad-REIC. In fact, treatment with Ad-REIC tended to suppress autophagy (Fig 4). This paradox is due to

the enhancement of mTOR signaling induced by Ad-REIC (Fig. 5). This mechanism of Ad-REIC may result in synergistic effects with chemotherapy and the ability to overcome drug resistance.

As indicated above, Ad-REIC has the potential to improve treatments for drug-resistant pancreatic cancer by suppressing autophagy. Many patients cannot receive effective treatment because pancreatic tumors often acquire drug resistance. Ad-REIC therapy may provide such patients a ray of hope.

In a prostate cancer study, REIC/Dkk-3 was reported to induce immunological effects that activate anti-tumor immune responses [38]. Although REIC/Dkk-3 has been reported to induce apoptosis via the c-Jun N-terminal kinase pathway in cancer cells [13], normal cells do not exhibit apoptosis due to the REIC/Dkk-3 expression. The REIC/Dkk-3 expression induces the production of interleukin (IL)-7 via the p38-STAT1-IRF-1 pathway [17, 39]. IL-7 is considered to stimulate systemic immune responses and exert anti-tumor effects. Such immune responses may lead to potential therapeutic effects on metastasis of pancreatic cancer. In the present study, we used immune-deficient mice that were not suitable for the study of anti-tumor immunity. As Ad-REIC is a promising gene therapy for pancreatic cancer patients, the development of a delivery method is important for the treatment of pancreatic cancer. Although it is

difficult to deliver viral gene therapy reagent to pancreatic cancer cells, endoscopic ultrasound (EUS)-guided approaches to treating pancreatic diseases have recently been generalized. EUS-guided approaches may be applied to new local therapeutic modalities [40].

## **Conclusion**

We demonstrated that REIC/Dkk-3 exerts anti-tumor effects in pancreatic cancer and other cancer cells. REIC/Dkk-3 gene therapy is a promising approach for overcoming pancreatic cancer.

## **Acknowledgements and potential conflicts of Interest:**

Momotaro-Gene, Inc. holds the patents for the Ad-REIC agent and developed the agent as a therapeutic cancer medicine. Dr. Kumon, Dr. Nasu and Dr. Watanabe own stock in Momotaro-Gene, Inc.

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### Figure legends

Figure 1. The REIC/Dkk-3 expression in the pancreatic cancer tissues.

The REIC/Dkk-3 protein level was determined using an immunoblot analysis. The cell lysate obtained from the ASPC1 cells treated with Ad-REIC (20 MOI) was used as a positive control. Immunoblotting for  $\beta$ -actin was performed to verify equal loading. Representative blots of more than three independent experiments are shown (A).

The pictures show the results of the REIC/Dkk-3 expression analysis of the human pancreatic tissue samples (top left: the islets of Langerhans in a tumor-free section scored (+++), top right: pancreatic cancer cells scored (-), bottom left: pancreatic cancer cells scored (+), bottom right: pancreatic cancer cells scored (++)). Representative pictures are shown. Scale bars, 50  $\mu$ m (B).

Figure 2. Effects of Ad-REIC in the pancreatic cancer cell lines.

The degree of cell proliferation and apoptosis was evaluated using an MTT assay and Hoechst staining, respectively. An MTT assay was performed to determine whether Ad-REIC influences cell growth in the pancreatic cancer cell lines ASPC1 and MIAPaCa2. The concentrations of Ad-LacZ and Ad-REIC were diluted to 100 MOI and 300 MOI. 1 mM of GEM was also used to determine whether REIC gene therapy

increases the effects of standard systemic chemotherapy against pancreatic cancer (A).

Hoechst 33342 staining was performed to evaluate the degree of apoptosis.

The results are presented as the mean  $\pm$  SE (n=5) (B).

Figure 3. Immunoblot analysis of the c-jun N-terminal kinase signaling pathway.

An immunoblot analysis was performed to evaluate signals of the c-Jun N-terminal kinase pathway in Ad-REIC-transduced ASPC1 cells. The immunoblot analysis was performed using antibodies against IRE1, phospho-IRE1, phospho-JNK, phospho-c-Jun and  $\beta$ -actin. Immunoblotting for  $\beta$ -actin was performed to verify equal loading. Representative blots of more than three independent experiments are shown.

Figure 4. Effects of Ad-REIC on autophagy.

ASPC1 cells treated with Ad-LacZ and/or Ad-REIC in the presence of rapamycin (500  $\mu$ M). The cells were stained with autophagy detection reagent and Hoechst33342 and examined under fluorescence microscopy (A). The number of cells exhibiting autophagy was counted (B).

Figure 5. Immunoblot analysis of the mTOR pathway.

An immunoblot analysis was performed to evaluate signals of the mTOR pathway in Ad-REIC-transduced ASPC1 cells. The immunoblot analysis was performed using antibodies against phospho-mTOR (s2448), phospho-mTOR (s2481), phospho-p70S6K and  $\beta$ -actin. Immunoblotting for  $\beta$ -actin was performed to verify equal loading. Representative blots of more than three independent experiments are shown.

Figure 6. Effects of Ad-REIC on tumor growth in the mouse pancreatic cancer xenograft model.

The inoculated mice were divided into five groups (control, Ad-REIC, Ad-LacZ, Ad-REIC+GEM and Ad-LacZ+GEM), and the tumor volume was measured as described in the Materials and Methods section. The data represent the mean  $\pm$  SE of more than three independent experiments performed in ten mice (A).

The immunoblot analysis was performed to evaluate the transduction efficiency of Ad-REIC in the tumor tissue. The tumor tissue samples were collected 72-h after the injection of Ad-REIC or Ad-LacZ. Immunoblot analysis was performed with anti-REIC/Dkk-3 antibody, anti- $\beta$ -gal antibody, and anti- $\beta$ -actin antibody. Immunoblotting for  $\beta$ -actin was performed to verify equal loading. Representative blots of more than three independent experiments are shown (B).